USING A THYROID PEROXIDASE INHIBITION ASSAY TO SELECT CHEMICALS FOR TESTING IN AMPHIBIAN-BASED THYROID TOXICITY ASSAYS

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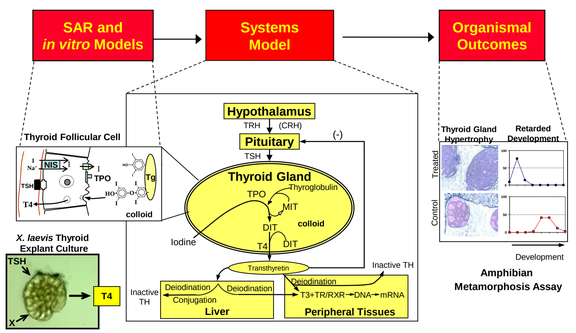
ABSTRACT

Within the context of developing an amphibian-based hypothalamic-pituitary-thyroid axis systems model to predict the potential for chemicals to produce thyroid toxicity, there was a need to develop an initial screening tool to select chemicals for testing. One mechanism by which chemicals can affect this axis is via direct inhibition of thyroid peroxidase (TPO), the enzyme responsible for thyroid hormone production. TPO catalyzes the iodination and coupling of tyrosines that are ultimately released from the thyroid gland as thyroid hormone. TPO activity can be measured using a guaiacol oxidation reaction which is a surrogate for the tyrosine coupling reaction. Microsomes prepared from porcine thyroid glands were incubated in the presence of guaiacol and test chemical to determine the inhibitory potency of chemicals on this enzyme. The reaction was initiated with hydrogen peroxide and the change in absorbance at 470 nm was measured. Methimazole and propylthiouracil, two model inhibitors of TPO, produced dose-related inhibition of TPO activity. Methimazole was more potent than propylthiouracil by an order of magnitude. The concentration that inhibited enzyme activity by 50% (IC50) was 1.3 µM and 11 µM, for methimazole and PTU, respectively. Perchlorate, which inhibits thyroid hormone production by inhibition of iodine uptake into the thyroid gland, was also positive in this assay but much less potent, with an IC50 of 13 mM; a 1000-fold greater concentration than that of PTU. A series of seven alkylphenols were tested ranging from phenol with no alkyl chain through nonylphenol. None of these alkylphenols inhibited TPO when tested at concentrations up to 3600 µM. Further development and testing with this assay to screen more chemicals and to determine their relative potency compared to methimazole is being conducted. These results represent the initial stages of an effort to determine structure activity relationships for TPO inhibition by xenobiotic chemicals and to select candidate chemicals to test in an amphibian thyroid gland explant culture system and in a tadpole metamorphosis assay.

Thyroid-axis Systems Model



A framework to organize and interpret toxicological data from molecular to organismal levels, and serves as a basis for developing tools for predicting toxicity

OBJECTIVES

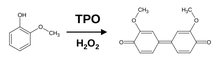
- Develop a rapid assay for assessing inhibitory effect of chemicals on thyroid hormone release
- Define inhibition dose responses for model inhibitors and estimate potency
- Test chemicals within defined chemical classes for their potential to inhibit thyroid hormone synthesis and to use this information to develop predictive models for thyroid hormone inhibition

METHODS

Guaiacol Oxidation Assay

The TPO inhibition assay is based upon guaiacol oxidation. The reaction is a surrogate for the TPO catalyzed coupling of iodo-tyrosines that occurs in the thyroid gland.

Guaiacol Oxidation[1]



Abs (470 nm)

- Microsomes were prepared from porcine thyroid glands (obtained from Hormel, Austin, MN) in 0.2 M phosphate buffer (pH 7.4, 5% glycerol).
- Microsomal protein (~ 150-200 µg total protein), guaiacol (35mM), and chemical were added to wells of a 96-well plate
- Initial absorbance at 470 nm measured (BioRad, 3550 Microplate Reader)
- H_2O_2 (300 µM) was added to initiate the reaction and absorbance at 470 nm measured at 60s
- Activity was calculated as the change in absorbance / min / mg protein
- A full dose-response curve for methimazole was generated in parallel with each chemical as a positive control for inhibition
- Inhibition potency of chemical was compared to potency of methimazole
- Relative Inhibitory Potency =

  molar IC_{50} methimazole / molar IC_{50} Chemical X

- Solubility of chemicals in the reaction matrix was determined indirectly at the conclusion of the experiment by nephelometry (light scatter) (Nepheloskan Ascent, Thermo Electron Corp., Vantaa, Finland).

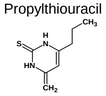
Chemicals

Model T4 Synthesis Inhibitors

TPO Inhibitors

- Methimazole

- Propylthiouracil (PTU)

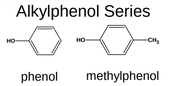


NIS Inhibitor

- Perchlorate

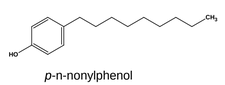
Test Chemicals

Alkylphenol Series



phenol methylphenol

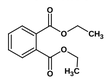
to ...



p-n-nonylphenol

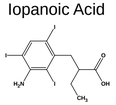
Selected for testing based upon TPO inhibition by hydroxylated-flavonoids and reported TPO inhibition by nonylphenol[2,3]

Phthalates



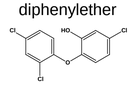
n-diethyl-phthalate

Iopanoic Acid



Type II deiodinase inhibitor

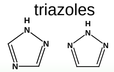
OH-polychlorinated diphenylether



triclosan

Other Chemical Classes Currently Under Investigation

triazoles

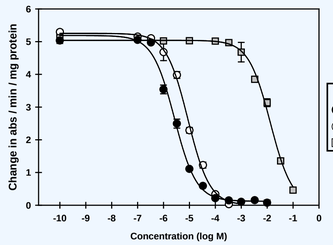


isothiazolinones

triazines

RESULTS

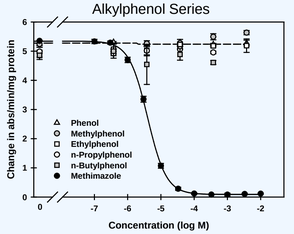
Model Thyroid Synthesis Inhibitors



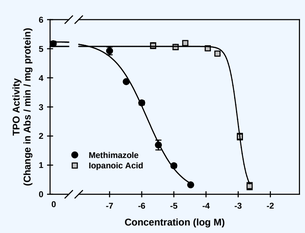
	IC50 (µM)
Methimazole	2.7
PTU	8.8
Perchlorate	13,400

Methimazole is the most potent of the model T4 synthesis inhibitors in the TPO inhibition assay. Perchlorate had very low potency for TPO inhibition, but was the most potent of the three inhibitors in the *ex vivo* and *in vivo*[4,5] assays. The primary mechanism of action for perchlorate inhibition of T4 synthesis is by inhibiting iodide uptake into the follicular cells.

Test Chemicals



All alkylphenols tested exhibited no inhibition of TPO activity



IOP exhibited full inhibition of TPO activity, although low potency compared to methimazole

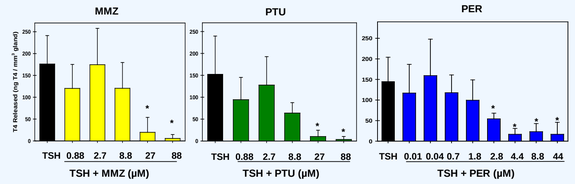
Table 1. Thyroid peroxidase inhibition as determined in the guaiacol oxidation assay.

Chemical	IC50 (µM)	Relative Potency	Highest Test Conc. (µM)[a]	Chemical Class
Model T4 Synthesis Inhibitors				
methimazole	2	1		thiamazole
6-propylthiouracil	8.8	0.3		thiamazole
perchlorate	13,000	0.0002		inorganic anion
Test Chemicals				
phenol	inactive	--	3670	alkylphenol
methylphenol	inactive	--	3670	alkylphenol
ethylphenol	inactive	--	3670	alkylphenol
n-propylphenol	inactive	--	367	alkylphenol
n-butylphenol	inactive	--	367	alkylphenol
n-octylphenol	inactive	--	3670	alkylphenol
nonylphenol (mixed branch)	inactive	--	3670	alkylphenol
nonylphenol (straight chain)	inactive	--	3670	alkylphenol
diethylphthalate	inactive	--	3670	phthalate
benzylbutylphthalate	inactive	--	3670	phthalate
Triclosan	inactive	--	3670	OH-polychlorinated diphenyl ether
iopanoic acid	760	0.0015	2260	iodobenzenes

[a] Highest test concentration is the highest concentration tested with no observed precipitate formation or increased light scattering as measured by nephelometry.

Inhibition of TSH-Stimulated T4 Release by Cultured Thyroid Glands

Thyroid glands from *X. laevis* tadpoles (NF stage 59) were cultured in L-15 media in the presence of 2000 ng TSH/ml alone or TSH and graded concentrations of chemical. Media was collected and analyzed by RIA for T4.

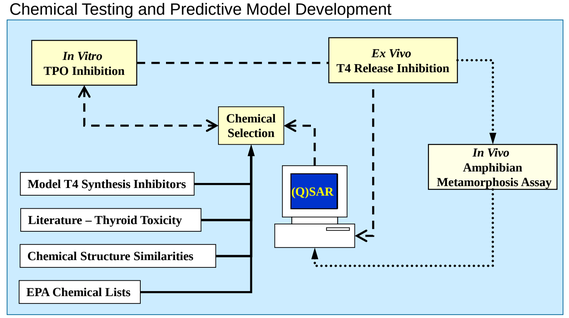


The potency of methimazole and PTU for inhibiting TPO activity is similar to that for inhibiting TSH stimulated T4 release from thyroid gland explant cultures. Perchlorate is more potent in this assay because it can inhibit T4 synthesis in the cultured thyroid glands by inhibiting iodide uptake.

Conclusions and Future Research

- The results of the TPO assay correspond well with the *ex vivo* assay system when TPO inhibition is the primary mechanism of action of the chemical. Differences in potency may indicate different, or multiple, mechanisms of thyroid hormone synthesis inhibition
- Chemicals that show inhibitory activity in the TPO inhibition assay need to be tested further in the *ex vivo* thyroid gland culture assay to confirm their effects on thyroid hormone synthesis and release
- The TPO assay can be used to rapidly screen chemicals for further testing in the higher level thyroid toxicity assays and can be used to begin to develop predictive models incorporating structure activity relationships between chemical structure and T4 synthesis inhibition
- This suite of assays can be an effective tool to determine the capacity of previously untested or unsuspected classes of chemicals to disrupt normal thyroid hormone production

Chemical Testing and Predictive Model Development



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References:

1. Doerge et al. 1997. *Anal. Biochem.* 250, 10-17.
2. Divi and Doerge, 1996. *Chem. Res. Toxicol.*, 9, 16-23.
3. Schmutzler et al. 2004. *Toxicology* 205, 95-102.
4. Degitz et al. 2005. *Toxicol. Sci.*, 87, 353-364.
5. Tietge et al. 2005. *Environ. Toxicol.Chem* 24, 926-933.